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User Manual

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Cellular Activation of Signaling ELISA

Profiling Pathway Activation via Cell-Based Detection of Protein Phosphorylation

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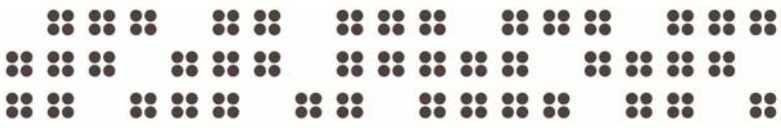

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Cellular Activation of Signaling ELISA CASE™ Kit

Profiling Pathway Activation Via Cell-Based Detection of Protein Phosphorylation

USER MANUAL

ORDERING INFORMATION AND TECHNICAL SERVICE

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LIMITED PRODUCT WARRANTY

This product is intended for research purposes only and is not intended for drug or diagnostic purposes or for human use. This warranty limits our liability to replace this product in the event the product fails to perform due to any manufacturing defect. SuperArray Bioscience Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose. SuperArray Bioscience Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use this product.

NOTICE TO PURCHASER

The purchase of a CASE™ Kit includes a limited, nonexclusive license to use the kit components for research use only. This license does not grant rights to use the kit components for reproduction of any kit component, to modify kit components for resale or to use a CASE™ Kit to manufacture commercial products without written approval of SuperArray Bioscience Corporation. No other license, expressed, implied or by estoppel, is granted.

I. BACKGROUND AND INTRODUCTION:

As an intracellular signal transduction pathway involving a protein kinase cascade is activated by an extracellular stimulus, the extent of phosphorylation of an upstream regulatory protein increases. Monitoring the phosphorylation status of this protein helps verify whether the stimulus activates the pathway and helps determine the effect of various treatments, inhibitors, or activators on that activation.

Two traditional methods are widely used to measure the phosphorylation status of a protein, Western blot analysis with phospho-specific antibody or [^{32}P] incorporation followed by gel electrophoresis. Both methods are tedious, require a large amount of cells and treatment reagents, and are not suitable for high-throughput studies.

The Cellular Activation of Signaling ELISA (CASE™) Kits employ a cell-based ELISA that directly measures protein phosphorylation on 96-well cultured cells. The CASE™ Kits include a complete antibody-based detection system for colorimetric quantification of the relative amount of phosphorylated protein and total target protein. This simple and efficient cell-based protein phosphorylation assay eliminates the need to prepare cell lysates and to perform a Western blot or the need to use r-[^{32}P]-ATP for *in vitro* kinase assays. The entire assay occurs directly in cell culture wells without sacrificing specificity, reproducibility, or sensitivity. With its 96-well plate format, the CASE™ assay requires very small amounts of cells and treatment reagents, and the assay procedure is compatible with high-throughput studies. These features make the CASE™ kits especially suitable for screening compounds that activate or inhibit important signaling pathways.

In the CASE™ assay, cells are seeded into 96-well plates. After your experimental treatment, the cells are fixed to preserve any activation-specific protein modification, such as phosphorylation. Two primary antibodies are included in the kit. One antibody recognizes only the activated (phosphorylated) form of the specific target protein, while another recognizes the specific target protein regardless of its activation state. Following incubation with primary and secondary antibodies, the amount of bound antibody in each well is determined using a developing solution and an ELISA Plate Reader. The absorbance readings are then normalized to relative cell number as determined by a cell staining solution. The amount of phosphorylated protein, once normalized to the amount of total protein, is then directly related to the extent of downstream pathway activation.

Features and Advantages of the CASE™ Kits:

- Easy, quantitative, and non-radioactive protocol with < 3 hours hands-on time
- No loss of activation state during procedure
- Cell-based assay: No cell extraction, electrophoresis or Western blot
- Detect relative amount of total and phosphorylated target protein as well as cell number all at the same time

Method Reference: Versteeg HH, Nijhuis E, van den Brink GR, Evertzen M, Pynaert GN, van Deventer SJ, Coffer PJ, Peppelenbosch MP. A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. *Biochem J.* 2000 Sep 15;350 Pt 3:717-22.

CASE™ Kit Quick Reference Procedure:

(Assay time: < 3 hours of hands-on time)

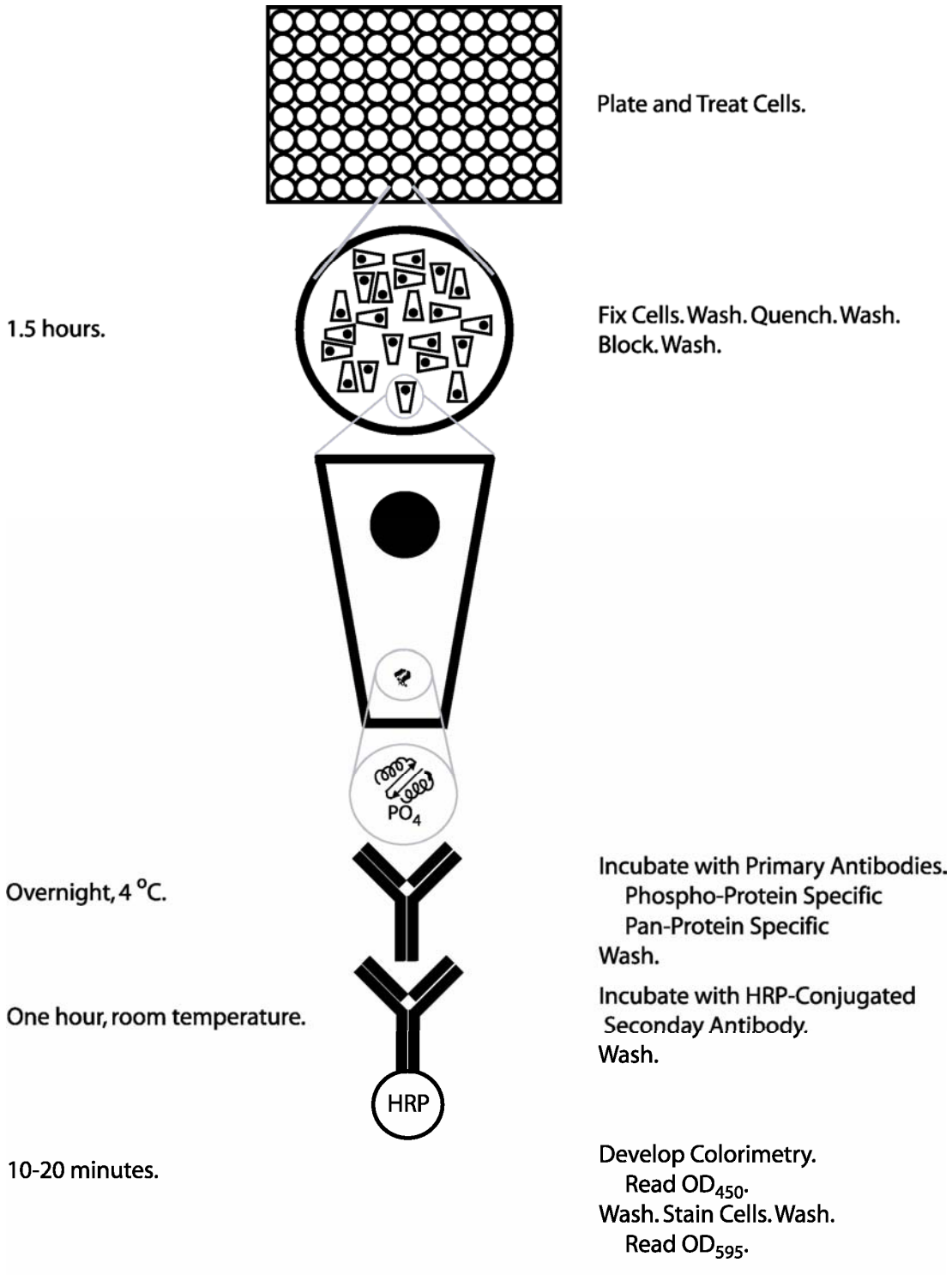

Figure 1: Overview of Cellular Activation of Signaling ELISA (CASE™) Kit Procedure.

II. Materials Provided:

This kit includes enough of the following reagents for 96 assays:

Box 1: Assay and Detection Reagents (Store at 4°C)	**Box 2: Antibodies**** (Store at -20°C)
COMPONENTS:	**COMPONENTS:**
10X Washing Buffer*	Anti-Phospho-Protein Specific Antibody
Antibody Dilution Buffer**	Anti-Pan-Protein Specific Antibody
Blocking Buffer, dry powder***	Secondary Antibody
Cell Staining Solution	
Developing Solution	
Stop Solution	
Washing Buffer Reservoir (4)	
Plate Sealing Film (4)	

* 10X Washing Buffer must be diluted to 1X with distilled H_2O before use.

** Each antibody stock must be freshly diluted into Antibody Dilution Buffer on the same day of the experiment. Use Dilution Factor specified for each antibody on its Product Information sheet. See page 9 for more details.

*** Just before use, dissolve powder in 30 ml 1X Washing Buffer by mixing well. Once dissolved, Blocking Buffer may be stored at 4 °C for up to two (2) weeks.

Shipping and Storage Conditions:

Box 1 is shipped at ambient temperature and should be stored at 4 °C.
Box 2 is shipped on dry ice and should be stored at –20 °C.

Shelf Life:

All reagents are stable as is if it is not used for 6 months after receipt of the kit and stored at the recommended temperature.

III. Additional Materials Required:

The following solutions are required for performing the CASE assay but are not included with the kit. These solutions must be prepared from materials obtained from other manufacturers before starting the CASE procedure:

1X Phosphate-Buffered Saline (PBS)

Dissolve 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7.4. Bring the final volume to 1 liter with distilled water. Dispense in convenient volumes and sterilize by autoclaving. Store at room temperature.

37% Formaldehyde

Molecular Biology Grade from any chemical supplier
For example: Sigma (Cat. No. F8775)

30% H_2O_2 (concentrated hydrogen peroxide)

Molecular Biology Grade from any chemical supplier
For example: Sigma (Cat. No. H1009)

10% NaN_3 (sodium azide):

Dissolve 10 g of sodium azide into 100 ml of ddH_2O.
Use appropriate caution – sodium azide is a hazardous substance.

1% SDS (sodium dodecyl sulfate)

Dissolve 1 g of sodium dodecyl sulfate into 100 ml of ddH_2O.
Store at room temperature.

0.01% poly-L-Lysine (for suspension cells only)

Molecular Biology Grade from any chemical supplier
For example: Sigma (Cat. No. P4832)

The following supplies are also required but are not included in the kit and must be purchased separately:

96-well Tissue Culture Plates (for example, Corning Costar 3595)
Multi-channel Pipettor
Parafilm
Rocking platform
ELISA Plate Reader capable of reading at 450 and 595 nm.

IV. Protocol:

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING EXPERIMENT.

A. Working Solution Preparation:

The following working solutions should be freshly prepared from the stock solutions each time the experiment is performed using the following recipes:

1. Cell Fixing Buffer:

Prepare either 4% or 8% Cell Fixing buffer depending on the type of your cell culture.

Cell Fixing Buffer	Reagent	Volume for 96 wells
4% Fixing Buffer For **adherent** cells	1X PBS 37% Formaldehyde **Total Volume**	10.7 ml 1.3 ml **12 ml**
OR		
8% Fixing Buffer For **suspension** cells	1X PBS 37% Formaldehyde **Total Volume**	9.4 ml 2.6 ml **12 ml**

2. 1X Washing Buffer:

10X Washing Buffer must be diluted to 1X with distilled H_2O before use.

3. Quenching Buffer:

To 11.48 ml of 1X Washing Buffer, add 400 µl of 30% H_2O_2, and 120 µl of 10% NaN_3 for a final volume of 12 ml, enough for 96 wells.

4. Blocking Buffer:

Just before use, dissolve blocking buffer powder (provided in kit box 1) in 30 ml 1X Washing Buffer by mixing well. Once dissolved, Blocking Buffer may be stored at 4 °C for up to two (2) weeks.

5. Antibody Dilution:

Each antibody provided in the kit must be freshly diluted using Antibody Dilution Buffer each time the experiment is performed. The dilution factor for each antibody is specified on each kit's product information sheet.

	Volume of Diluted Antibody (Prepared in Antibody Dilution Buffer)		
Diluted Antibody	**8 assays**	**12 assays**	**96 assays**
Anti-Phospho-Protein Specific Antibody	500 µl	750 µl	6000 µl
Anti-Pan-Protein Specific Antibody	500 µl	750 µl	6000 µl
Secondary Antibody	2000 µl	3000 µl	24000 µl

Use the appropriate dilution factor for each antibody listed on the individual kit product information sheet.

> ***For example:*** *If the required dilution factor for an anti-phospho-protein specific antibody is 1:150 and 12 assays are to be performed, calculate the amount of stock antibody to dilute: 750 µl (from table above) / 150 (dilution factor) = 5 µl. Thus, 5 µl of antibody should be diluted into 750 µl of Antibody Dilution Buffer. Perform this calculation separately for both primary antibodies and the secondary antibody using their dilution factors and the total volumes in the table above.*

Dilute just enough of the antibodies as needed for the planned experiment. If you use the antibodies wisely, enough material is provided in the kit to process a total of 96 assays or, in other words, 96-wells with each primary antibody and a total of (2 x 96 =) 192 wells with the secondary antibody.

B. Cell Culture and Cell Preparation:

NOTE: A fresh and healthily growing cell culture is crucial for a successful CASE experiment. Only use cells growing in log-phase to set up a CASE experiment. If the cell culture is already too old (that is, if the cells are over-confluent, if the culture medium turns yellow in color, or if adherent cells have started floating to a significant extent), split the cells once into a fresh tissue culture flask or dish and fresh medium. Allow the cells to grow into log phase once more before seeding into a 96-well plate to set up the CASE experiment.

1. **Plan your experiment and your cell culture carefully. For example, see Figure 2.**

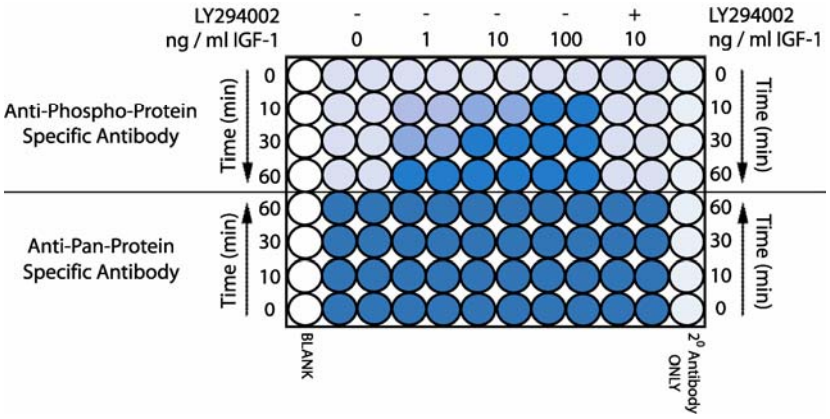

Figure 2: Example of how to plate cells for AKT CASE™ Kit. Seed cells and plan treatment in two identical sets of cell culture wells. In the example here, a 96-well plate is divided into two sets, and treatments are identical in both sets in a symmetrical fashion. Duplicate wells are used here for each dose and time point. Cells were treated with various concentrations (0, 1, 10, or 100 ng/ml) of IGF-1 with or without the kinase inhibitor LY294002 for 0, 10, 30, or 60 minutes. The 96-well plate was used in a CASE™ Kit Assay; half of the plate was treated with the anti-phospho-protein specific primary antibody, and half with the anti-pan-protein specific primary antibody. Eight wells of cells were not treated with primary antibody but only with secondary antibody (2° Antibody ONLY), and eight wells were not plated with cells but were treated with both primary and second antibodies (Blank). The different shades in 96-well plate represent theoretical signal intensities from the experiment.

a. Each treatment point (or assay determination) requires two sets of cells treated in an identical fashion. One set of cells will be incubated with the phospho-protein specific antibody to measure phosphorylated protein; the other set of cells will be incubated with the pan-protein specific antibody to measure total protein. Each experimental condition (*e.g.* time point, concentration point, *etc.*) should be performed in duplicate or triplicate to control for systematic variation.

b. You should also include wells for control purposes such as:

i. Blank wells (not seeded with any cells)
ii. Detection control wells (seeded with cells, only incubated with secondary but not primary antibody)
iii. Experimental control wells (seeded with cells but not experimentally treated)

NOTE: *If using non-adherent (that is, suspension) cells, pre-coat tissue culture wells by filling them with 10 μg/ml poly-L-Lysine, incubating at 37 °C for 30 minutes, and washing twice for 5 minutes with 1X PBS.*

c. Seed cells into a 96-well plate the day before your treatment, with the idea that the cell culture density should be at roughly 50-80 percent confluence on the day of your treatment and the CASE experiment.

> ***For example:*** *In the experiment outlined in Figure 2, cells are seeded at 2.0 x 10^4 cells per well on day 0 and allowed to sit and grow overnight. On day 1, the cells are starved in serum-free medium overnight. On day 2, the relevant wells of cells are pre-treated with the inhibitor for one hour followed by the IGF1 treatment as described in the figure legend. Then, the CASE assay protocol is performed as described below.*

For the majority of cell lines with a doubling time of 18-24 h, seeding an initial density of roughly 1.5 x 10^4 cells per well will yield the appropriate cell density by the time of the CASE assay. If you have not established a growth curve for your cell line in 96-well tissue culture plates, perform a pilot cell seeding experiment before seeding cells for the CASE experiment. Cell densities that are too high or too low at the time of the CASE assay give poor results. Re-seed cells if the cell growth or confluence conditions are not appropriate.

NOTE: *It is not necessary to plate cells in all 96 wells. Only add cells to the wells that you plan to use and prepare only as much antibody dilution as you need.*

2. Allow cells to grow overnight.
   Then, perform treatments as defined by your experiment on the next day.

3. Fix Cells:

   a. **For adherent cells,** remove the culture medium, and add 100 µl of **4%** Cell Fixing Buffer to each well.

   b. **For suspension cells,** directly add 100 µl of **8%** Cell Fixing Buffer to the 100 µl of culture medium in each well.

To minimize formaldehyde evaporation, seal the open plate with a piece of parafilm and place the lid onto the plate. Incubate for 20 minutes at room temperature.

WARNING: *Formaldehyde and its vapors are highly toxic. Always prepare and use formaldehyde solutions under a chemical fume hood and wear protective gloves and eyewear and a lab coat. Dispose formaldehyde waste according to your institution's hazardous waste disposal protocol.*

You may use the fixed cells right away or the fixed cells are also stable for several weeks in Cell Fixing Buffer. Seal with parafilm or in a zip lock bag and refrigerate at 4 °C. In this way, you can accumulate many plates in the refrigerator, and perform the CASE assays later when desired.

NOTE: *To minimize cell loss during all wash steps in the following protocol, never dispense liquid directly onto the cell surface. Instead, gently dispense liquid down the wall of cell culture wells. To remove wash solutions, flip the plate over a sink and then tap the plate gently onto a paper towel to remove any remaining liquid.*

4. Remove the Cell Fixing Buffer and wash cells twice with 200 µl of 1X Washing Buffer for 5 minutes each with rocking.

5. Remove Washing Buffer, add 100 µl of Quenching Buffer and incubate for 20 minutes at room temperature.

6. Remove Quenching Buffer and wash cells once with 200 µl of 1X Washing Buffer for 5 minutes with rocking.

7. Remove Washing Buffer, add 100 µl of Blocking Buffer and incubate 1 hour at room temperature.

C. Incubation with Primary and Secondary Antibodies:

1. Remove Blocking Buffer and wash cells once with 200 µl of 1X Washing Buffer for 5 minutes with rocking.

2. Remove Washing Buffer, and add 50 µl of diluted primary antibody (either the phospho-protein or the pan-protein specific antibody) to each appropriate well. See page 9 for antibody dilution instructions. To the negative control wells, add 50 µl of Antibody Dilution Buffer instead.

   To prevent evaporation, use a piece of the Plate Sealing Film provided in kit followed by a piece of parafilm to seal the plate. Then, cover the plate with its lid. Incubate overnight at 4 °C.

3. Remove the primary antibody, and wash cells twice with 200 µl of 1X Washing Buffer for 5 minutes each with rocking.

4. Remove the Washing Buffer, and add 100 µl of dilute secondary antibody to each well. Incubate for 1 hour at room temperature.

5. Meanwhile, transfer only the needed amount of Developing Solution (100 µl per well) into a clean Washing Buffer Reservoir and allow it to warm to room temperature while protected from light.

D. Colorimetric Detection:

1. Remove the secondary antibody, and wash cells twice with 200 µl of 1X Washing Buffer for 5 minutes each with rocking. Then, wash the cells once with 200 µl of PBS for 5 minutes with rocking.

2. Remove the PBS, and add 100 µl of Developing Solution (pre-warmed to room temperature) into each well. Protect plate from direct light.
   Incubate for 2 to 20 minutes at room temperature.

3. Monitor the blue color development until the darkest staining well turns a medium- to dark-blue. DO NOT OVERDEVELOP ANY WELLS.

4. Add 100 µl Stop Solution into each well to stop reaction. The Stop Solution turns the blue color to yellow.

   Note: Do not add Stop Solution to the Washing Buffer Reservoir that contained the Developing Solution. Use a clean reservoir for Stop Solution.

   Stagger the addition of the Developing and Stop Solutions in a manner that allows each well to develop for the same length of time.

WARNING: *The Stop Solution is corrosive. Always wear protective gloves and eyewear and a lab coat when handling this solution.*

5. Within 5 minutes, read absorbance at 450 nm on an ELISA Plate Reader. Blank the instrument according to the manufacturer's instructions.

E. Determine Relative Cell Number:

1. Remove the assay solution as described above.
   Wash the wells once with 200 µl of 1X Washing Buffer for 5 minutes with rocking.
   Wash the wells once with 200 µl of dH_2O for 5 minutes with rocking.
   Be sure to remove any excess liquid by tapping plate gently on paper towel.
   Air-dry at room temperature for 5 minutes.

2. Add 100 µl of Cell Staining Buffer to each well and incubate for 30 minutes at room temperature.

WARNING: *The Cell Staining Buffer is an intense stain. Avoid contact with skin and clothing.*

3. Wash the wells twice with 200 µl of dH_2O for 5 minutes each with rocking.

4. Add 100 µl of 1% SDS to each well and incubate on a rocker for 1 hour at room temperature.

5. Read absorbance at 595 nm on an ELISA Plate Reader.

   If the signals are greater than the linear range of the instrument, remove 50 µl of the 1% SDS solution from every well, and add 50 µl of dH_2O to each well. Repeat the reading of the absorbance at 595 nm on the ELISA Plate Reader.

6. Normalize the antibody reading to the relative cell number:
   Divide the OD_{450} readings for each well by its OD_{595} reading.

7. To determine the relative extent of target protein phosphorylation, normalize the phospho-protein specific antibody OD_{450}:OD_{595} ratio to the pan-protein specific antibody OD_{450}: OD_{595} ratio for the same experimental condition.

V. Troubleshooting Guide:

A. No or weak signal in wells incubated with either the phospho-protein specific or the pan-protein specific primary antibody:

1. Improper Plate Reader settings: Verify the wavelength and filter settings of the ELISA Plate Reader.
2. Cold Developing Solution: Warm the Developing Solution to room temperature before use.
3. Insufficient numbers of cells remain on the plate: Check cells on the plate using microscope and perform steps to Determine Relative Cell Number to measure how many cells remain on the plate.
4. Cells do not contain detectable levels of the target protein: Verify expression of the protein in your cell line by Western analysis using a pan-protein specific antibody.

B. No response observed to experimental treatments in wells incubated with phospho-protein specific antibody:

1. Cells culture conditions are not appropriate: When cells are not healthy (i.e. the cell culture is not fresh) or the cell density is too low or over confluent, cells may not respond to your treatment properly. Seed cells the day before your treatment so that the cell culture density is at roughly 50-80 percent confluence before your treatment. If you have not established a growth curve for your cell line in 96-well tissue culture plates, perform a pilot cell seeding experiment before seeding cells for the CASE experiment. For the majority of cell lines with a doubling time of 18-24 h, seeding an initial density of roughly 1.5 $X10^4$ cells per well will yield the appropriate cell density by the time of the CASE assay.
2. Treatment does not activate target protein: By Western analysis using a phospho-protein specific antibody, verify that your treatment conditions are actually activating the target protein. Use a well-established treatment known to induce the specific protein phosphorylation event as your positive control.
3. Insufficient antibody sensitivity: If the absorbance readings in the wells incubated with phospho-protein specific antibody are significantly above background but do not change in response to your treatment, then the ability to detect smaller changes can be improved by decreasing the concentration of anti phospho-protein specific antibody to half or reducing the antibody incubation time from overnight at 4 °C to 2 h at 37 °C.

C. Poor sensitivity (low absorbance readings for antibodies):

Primary antibody concentration is too low: For low protein expression levels (as determined by Western analysis), double the concentration of the primary antibodies and decrease the incubation volume by half. Take extra care in dispensing the smaller volumes and in sealing the plate to avoid evaporation.

D. The A_{450} readings for the wells incubated with the phospho-protein specific antibody are higher than the A_{450} readings from wells incubated with the pan-protein specific antibody:

The phospho-protein specific antibody is simply more sensitive than that the pan-protein specific antibody. This phenomenon occurs in several of our CASE Kits. It does not affect your final assay results. Remember that the CASE Kits do not involve a calibration curve and therefore can only be used for relative measurement.

E. High background in all wells:

1. Developing time too long: Be sure to stop the colorimetric development as soon as the darkest positive wells turn a medium to dark blue.
2. Improper blocking and washing: Be sure that washing, quenching, and blocking steps were all performed according to the recommendations in the protocol. Remember to add the solutions carefully down the sides of the wells and to remove liquids by inverting plates over a sink and then tapping the plate gently on a paper towel.
3. Secondary antibody concentration is too high: Decrease the secondary antibody concentration to half or reduce the secondary antibody incubation time from 1 h to 30 min.

F. High absorbance in blank wells:

Stop Solution was contaminated with Developing Solution: Be sure to use a new and clean Buffer Reservoir for Stop Solution. Do not use a Buffer Reservoir that has been previously used to contain Developing Solution to hold Stop Solution.

G. Significant signal variation among replicate wells:

1. Cell loss during incubation and washing: Be sure to normalize antibody signals with the cell staining reading (A_{595}). A well with an A_{595} reading close to the reading of a blank well (lacking cells) indicates a complete loss of cells from that well. Omit this well from your data set.
2. Improper antibody incubation and washing: Be sure to fill all wells with the appropriate antibody and follow the recommendations for washing. Use a rocker in every wash step.

H. No antibody solution in wells after overnight incubation:

Be sure to seal the open plate with sealing tape then with a piece of parafilm and place the lid onto the plate. The plate may also be stored in a zip-lock or heat-sealed bag. Be sure to store the plate on a level, stable surface.

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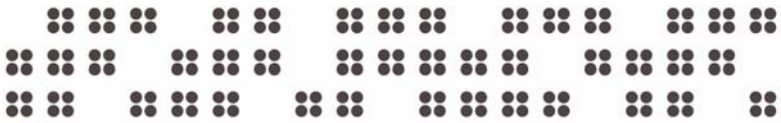

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